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Original Contribution

8-hydroxydeoxyguanosine suppresses NO production and COX-2 activity via Rac1/STATs signaling in LPS-induced brain microglia

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Abstract

Free 8-hydroxydeoxyguanosine (oh^{8}dG), a nucleoside of 8-hydroxyguanine (oh^{8}Gua), present in cytosol is not incorporated into DNA. However, nothing is known about its biological function when it presents in cytosol as a free form. We demonstrate here for the first time that oh^{8}dG inhibits lipopolysaccharide (LPS)-induced nitric oxide (NO) production and cyclooxygenase-2 (COX-2) activity, and both gene transcriptions in microglia. Furthermore, oh^{8}dG reduced mRNA levels of pro-inflammatory cytokine, such as IL-1β, IL-6, and TNF-α, in activated BV2 cells. We also found that oh^{8}dG suppressed reactive oxygen species (ROS) production through reduction of NADPH oxidase activity and blocked Rac1/STATs signal cascade. Finally, oh^{8}dG suppressed recruitment of STATs and p300 to the iNOS and COX-2 promoters, and inhibited H3 histone acetylation. Taken together, these results provide new aspects of oh^{8}dG as an anti-inflammatory agent.

Keywords: 8-hydroxydeoxyguanosine; NADPH oxidase; Rac1; STATs; iNOS; COX-2; Free radicals

Microglia, the major immune cells in brain, are activated commonly in response to variety of neurodegenerative and neuroinflammatory conditions and secrete reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory factors including nitric oxide (NO) and prostaglandin (PGs) [1–3]. NO produced by inducible nitric oxide synthase (iNOS) and PGs produced by cyclooxygenase (COX-2), have been implicated in a variety of pathophysiological conditions such as atherosclerosis, inflammation, and carcinogenesis [4–8]. iNOS and COX-2 gene expressions are regulated mainly at the transcriptional level through activation of STATs, NF-kB and MAPK [9 10].

JAK/STAT (janus kinase/signal transducers and activators of transcription) signaling pathway has been reported to mediate actions of growth factors, hormones, and cytokines [11]. Binding of their ligands to their respective receptors recruit and phosphorylate JAK. Phosphorylated JAKs provide the docking site for STATs, which in turn become phosphorylated. Phosphorylated STATs form dimers and translocate to the nucleus, which bind directly to target genes [12–14]. Recently, several studies have reported that JAK/STATs signaling mediate inflammatory responses in brain microglial cells [1 2]. However, it is unknown how JAK/STATs signal cascade is regulated in neuroinflammatory condition.

Abbreviations: ROS, reactive oxygen species; oh^{8}dG, 8-hydroxydeoxyguanosine; 8-oxo-dG, 8-oxo-deoxyguanosine; oh^{8}G, 8-hydroxyguanosine; 8-oxo-G, 8-oxo-guanosine; dG, deoxyguanosine; G, guanosine; oh^{8}dGTP, 8-hydroxydeoxyguanosine triphosphate; 8-oxo-deoxyguanosine triphosphate, 8-oxo-dGTP; STAT, Signal transducers and activators of transcription; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NADPH, nicotinamine adenine dinucleotide phosphate; LPS, lipopolysaccharide; TRITC, tetramethyl rhodamine isothiocyanate; FITC, fluorescein isothiocyanate; DAPI, 4′-6-Diamidine-2-phenylindole; DPI, dephenylene iodonium; NAC, N-acetyl-L-cysteine.

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It has been demonstrated that ROS are produced in response to cytokines and growth factors, contribute to cellular signaling, and play roles as second messengers [15–17]. Most of intracellular ROS are derived from NADPH oxidase. NADPH oxidase consists of two membrane-associated proteins ($gp91^{phox}$ and $p22^{phox}$) and at least four cytosolic proteins ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and a small GTPase Rac1 and Rac2). The enzyme complex is normally dormant, but becomes activated by variety of stimuli to catalyze NADPH-dependent reduction of oxygen to O_2^- [18 19]. Previous reports have shown direct links between NADPH oxidase, Rac activity and ROS production [20–23].

Guanine, a base in DNA is easily attacked by ROS to yield 8-hydroxyguanine (oh^8Gua), the most common oxidative DNA adduct, which is known to be mutagenic/carcinogenic and is used as a marker of oxidative stress [24–26]. However, mammalian cells have multiple repair systems such as base excision repair (BER) or nucleotide excision repair (NER) mechanism. oh^8Gua in DNA is mainly eliminated by both BER and NER mechanisms [27–29]. We previously reported when oh^8Gua exists in cytosol as a free base, not parts of DNA, its degradation consequently protects the bases in DNA from attack by ROS. Its protective action is much stronger than those of well-known ROS scavengers such as mantiol, DMSO, t-butyl alcohol, azide and formate, indicating that oh^8Gua is a stronger antioxidant [30]. This further implies that oh^8Gua may have anti-inflammatory effects, because antioxidant agents are considered as anti-inflammatory drugs [18]. 8-hydroxydeoxyguanosine (oh^8dG), nucleoside of oh^8Gua, is generated from 8-hydroxy-Gua-containing oligomer by NER or from cytoplasmic oxidized nucleotides, 8-hydroxy-dGTP (oh^8dGTP) [27–29]. Recently, we reported that exogenous oh^8dG is not incorporated into DNA, and that it does not play as any oncogenic reagent [31 32]. However, biological function of cytosolic oh^8dG remains unknown.

In this study, we explored the roles of oh^8dG in anti-inflammatory action by virtues of antioxidant property of oh^8Gua. We demonstrated here for the first time that oh^8dG significantly suppressed lipopolysaccharide (LPS)-induced NO production and COX-2 activity by decreased iNOS and COX-2 expression. oh^8dG also reduced transcriptions of pro-inflammatory cytokines. In addition, oh^8dG suppressed ROS production through inhibition of NADPH oxidase activity, Rac1 activity, and inhibited Rac1/STATs signal cascade. Therefore, this study provides new insights that oh^8dG may function as an anti-inflammatory agent in brain microglia.

Materials and methods

Animals

Male C57BL/6 (22-25 g/ individual) mice were housed at 23±2°C with a 12 hr light-dark cycle (light on from 08:00 to 20:00), and fed food and water *ad libitum*. The animals were allowed to habitate to housing facilities for 1 week before the experiments, and efforts were made to limit distress to the animals. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) in College of Medicine, Seoul National University, Korea.

Materials

LPS (Escherichia coli serotype 0111: B4), guanosine (G), deoxyguanosine (dG), oh^8dG, 4′-6-Diamidine-2-phenylindole (DAPI), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), nicotinamine adenine dinucleotide phosphate (NADPH), N-acetyl–L-cysteine (NAC) and dephenylene iodonium (DPI) were purchased from Sigma Chemical Co. (St Louis, MO). Anti-STAT1 antibody, anti-STAT3 antibody, anti-phospho-STAT1 antibody, anti-phospho-STAT3 antibody, anti-iNOS antibody and anti-COX-2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-JAK2 antibody, anti-phospho-JAK2 antibody, GST-PAK-PBD and anti-Rac1 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). oh^8G and COX inhibitor screening assay kit were purchased from Cayman Chemical (Ann Arbor, MI). Anti-oh^8dG antibody was purchased from Japan Institute for the control of aging (Shizuoka, Japan). Anti-OX-42 antibody was purchased from Serotec (Oxford, UK) and biotinylated secondary antibody, avidin and biotinylated horse radish peroxidase (HRP) complex were purchased from Vector Labs. (Burlingame, CA). TRITC- and FITC-conjugated mouse/rabbit IgG antibody were purchased from Jackson ImmunoResearch (USA). Fc-OxyBurst Green assay reagent was purchased from Molecular Probes Inc. (Eugene, OR).

Intrastriatal injection

Mice were randomly assigned to three groups; control group (PBS vehicle injected, n=6), LPS-treated group (n=6), and oh^8dG plus LPS-treated group (n=6). The previously described method [33] was modified as follows for the intrastriatal administration of drugs. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg), placed on a stereotaxic apparatus (Myneurolab, St. Louis, MO). PBS, oh^8dG (8.0 μg in 2.0 μl PBS) or LPS (1.0 μg in 2.0 μl PBS) was injected into the right striatum (stereotaxic coordinates in mm with reference to bregma: AP, +0.7; ML, +2.0; DV, −3.5) using a 10 μl Hamilton microsyringe with a 26-gauge needle. oh^8dG was administrated by intrastriatal injection for 1 hr before LPS injection. Control mice were injected with an equal volume of PBS vehicle.

Immunohistochemistry / immunofluorescence analysis

After deep anesthesia, the mice were perfused with fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed at 4°C overnight, transferred to 30% sucrose in PBS for 48 hr, and then coronal sections (30 μm-thickness) were prepared by cryostat. The immunohistochemical analysis of floating sections was performed as previously described [34]. Briefly, sections were incubated for 30 min with 3% H_2O_2 in 0.1 M PBS (pH 7.4), blocked with solution containing 5% normal goat or horse